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APPENDIX A

EXHIBIT 1

Antisense approaches to cancer gene therapy

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Recent advances in the use of oligodeoxynucleotide and plasmid-derived RNA as antisense agents of special relevance to cancer gene therapy are summarized with emphasis on agents and systems which have lead to clinical trials and/or regression of established tumors in animal model systems. Transformed cell lines bearing plasmids and viruses designed for the transcription of antisense RNA have the advantage that they can be characterized thoroughly and the effects of antisense RNA on target gene expression and phenotype can be studied easily *in vivo*. Promising results make the considerable efforts of applying oligodeoxynucleotides in whole animals and in clinical trials more plausible. Conversely, oligodeoxynucleotide experiments which yield promising results in tissue culture can be generalized to the *in vivo* setting by development of clones of cells bearing plasmid-derived antisense RNA against the same target. Several examples of the concordant results for oligodeoxynucleotide and plasmid-derived antisense RNA against the same target are considered. The importance of examination of antisense effects in syngeneic and immunocompetent hosts is illustrated by studies of insulin-like growth factor and insulin-like growth factor receptor where tumor regression and protection against tumor formation have been observed for particular cell types in defined settings.

Key words: Antisense RNA; cancer; gene therapy; tumor regression.

The antisense approach to gene therapy comprises the use of a substance to intervene in the natural processing of genetic information in the cell,[1–3] particularly when an aberrant gene is causing disease. Generally these substances will be nucleic acids, and will contain genetic information in their base sequences. The substance could be produced biologically, ie, by the use of a suitable vector, such as a plasmid or attenuated virus,[4] in the form of an antisense MRNA that binds by complementary Watson-Crick base pairing to the natural sense mRNA. Alternatively, the antisense substance in question will be synthesized chemically. The simplest form of such a "genetic drug" is a small piece of DNA, an oligodeoxynucleotide. These two antisense approaches are shown schematically in Fig 1. A related approach involves ribozymes which are catalytic ribo-oligomers that degrade a complementary mRNA after binding via antisense base pairing (Fig 2).

As well as antisense, there are other related approaches that—instead of inhibiting translation—are designed to inhibit transcription. These are (1) triplex formation, in which an oligomer is targeted to the gene directly and, by binding in the major groove of DNA, forms a triple-stranded helix, and (2) the use of transcription factor decoys that are duplexes designed to bind to a particular transcription factor thereby preventing its normal function (Fig 3). There are many similarities as well as differences between each of these strategies. We will first consider the applications and development of synthetic oligomers, and then the meth-

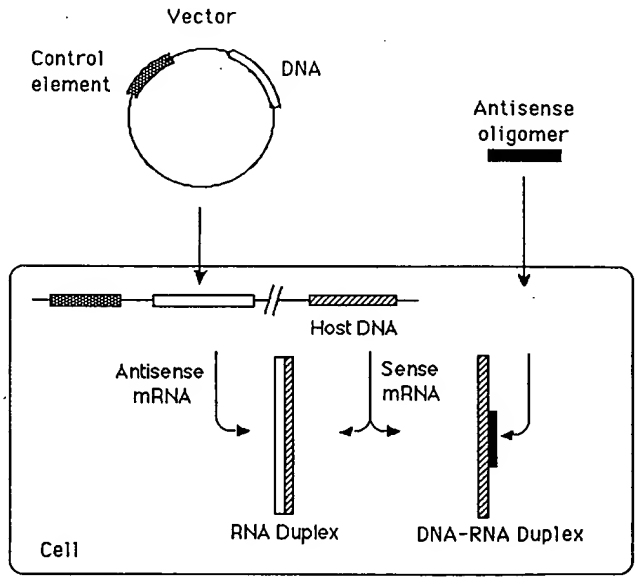

Figure 1. Schematic of translation arrest either endogenously by an antisense mRNA transfected via a suitable vector, or exogenously by an antisense oligodeoxynucleotide.

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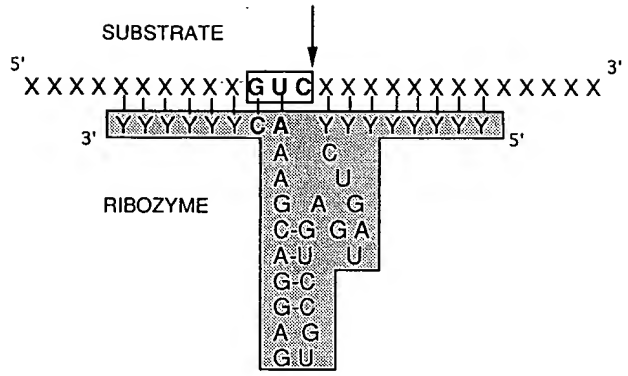

Figure 2. Schematic of a ribozyme; the bases labeled Y are the antisense sequence. The arrow indicates the point of scission on the target mRNA by the active site region.

ods that utilize endogenous antisense mRNA production.

ANTISENSE OLIGODEOXYNUCLEOTIDES

Cell uptake

In all of these genetically based strategies the oligomer used must be able to enter the cell to interact with the genetic machinery, and consequently they must cross the cell membrane. Since these substances are all oligomeric (single or double stranded) and have negatively charged phosphate backbones, their charged nature represents a potential problem. However, there is now ample evidence that these compounds do enter the cell, most likely by endocytosis,[5] and they do reach their target. It should be remembered that since the target (such as mRNA) is present in nanomolar concentrations, only a very small proportion of the extracellular or intracellular drug is needed to be effective. Means to improve the cellular uptake of these substances will be discussed below.

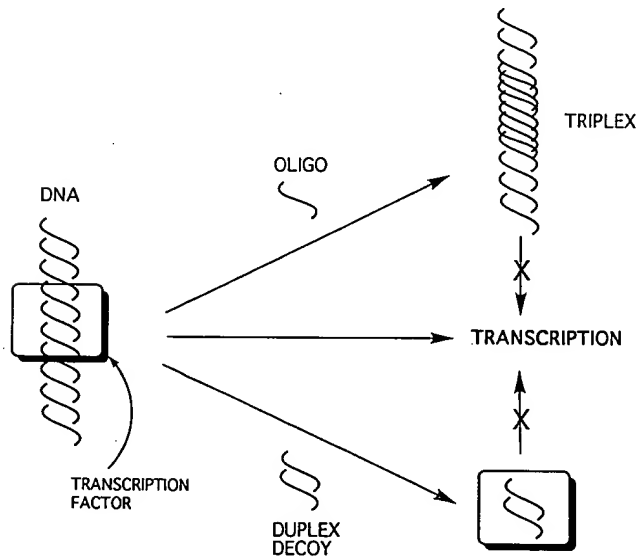

Figure 3. Schematic of transcription arrest either by triplex formation or by transcription factor decoys.

Oligodeoxynucleotide analogs

Cells and organisms protect themselves against foreign DNA and RNA by producing nucleases that degrade phosphodiesters.[6] To transform an oligodeoxynucleotide into an effective drug it is necessary to chemically modify it so that it is resistant to these nucleases. The simplest way of doing this is to modify the phosphodiester backbone, such that a group or atom is substituted for one of the phosphate oxygens, thus forming a resistant bond. The two most common such analogs are the methyl-phosphonate, using a methyl group, or phosphorothioate, in which a sulfur atom is used as a substituent (Fig 4). There are many other ways of modifying the basic mononucleotide unit, and these have recently been reviewed.[3] An interesting new analog is that in which the whole deoxyribose-phosphate backbone is replaced with a peptide-like backbone, to form a series of compounds called the peptide nucleic acids or PNAs (Fig 4).[7] These compounds herald the development of a whole new category of synthetic gene-mimetic substances.[3]

Pharmacokinetics

Several studies of pharmacokinetics of oligodeoxynucleotides have been reported in both mice and rats. Since the phosphorothioate analog is water soluble and is easily synthesized it has been the most widely used and tested for its pharmacokinetic and toxicological properties.[8–10] The results indicate that these oligomers can be expected to provide sufficient tissue concentration to be effective drugs. Notably, the slower plasma clearance process after single dose injection has a half-life of many hours (eg, mean 34 hours in rats[10]).

The Anti-gene approach

Since DNA itself is a tightly bound double helix, it is unlikely that a single-stranded oligomer will be able to disrupt the duplex and bind in an antisense manner. Consequently advantage is taken of the fact that in the B-form of DNA the large major groove leaves enough space for a third strand to bind to form a triple helix or triplex. The specificity for this binding is not Watson-Crick pairing, since the base pair is already formed, but is Hoogsteen (or anti-Hoogsteen) hydrogen-bonded interactions of a third base with the already formed pair.[11] The efficacy of this approach has been demonstrated using catalytic groups, conjugated to the end of an oligomer, that can degrade the target DNA in one (or a few) sites,[12] and it has also been demonstrated to work *in vitro*.[13] However, the development of this approach has not yet proceeded beyond the preclinical stage.

Transcription factor decoys

The idea of sequestering transcription factors, or in general proteins with a binding site for a specific DNA sequence, has been proposed. The use of a duplex of

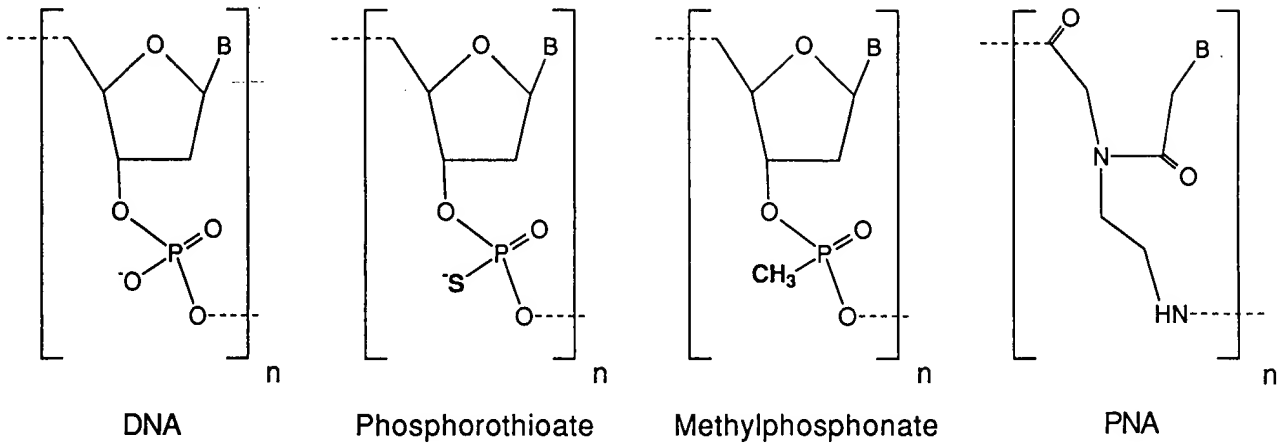

Figure 4. Comparison of monomeric units making up the DNA phosphate backbone structures in natural and "non-hydrolyzable" synthetic drivatives.

phosphorothioate oligomers has been reported.[14] Alternatively, to overcome the problem of degradation by exonucleases, and the potential dissociation of the duplex *in vivo*, cyclic oligonucleotides were developed.[15] These cyclic oligomers have interesting properties, one of which is to bind to a single-stranded region to form a triplex, so they might be useful to select for a single stranded target nucleic acid *in vivo*. However, the non-self–complementary single stranded bridge regions are susceptible to endonuclease degradation. To overcome this cyclic oligonucleotides with nonnucleotide bridges have been synthesized in which the ends are connected 3′ to 5′.[16] These may be effective decoys because they are highly stabilized; they are prepared by ligation after synthesis with the bridges present. This locks the duplex in place and since the bridges prevent unwinding, very high melting temperatures are observed, and the tightly wound helix is also resistant to endonucleases.[16] So far no actual applications of these decoys have been reported.

Antisense

By far the most developed topic in this area of drug development, antisense has the attractive simplicity of an elegant idea. But, in practice, as with any therapeutic modality, problems arise, notably; (a) degradation of the oligomer (even chemically modified analogs are eventually degraded *in vivo*); (b) inefficient cell uptake; (c) nonspecific binding (binding of oligomers to cell membranes and to proteins); (d) nonspecific cleavage of mRNA (due perhaps to partial or transient base pairing of the oligomer to sequences other than the target sequence). Nevertheless, some 36 applications of phosphorothioate oligonucleotides have been reported,[3] and these represent a considerable body of evidence that antisense is a useful approach for delineation of gene function, apart from its therapeutic potential.

Ribozymes

Ribozymes are essentially antisense oligomers in which the antisense portion flanks an RNA active site that cleaves a bound RNA. Ribozymes exist naturally, but have been adapted to degrade a specific mRNA sequence.[17] Note that although ribozymes are generally considered to be composed entirely of RNA, it is possible to synthesize analogs in which many of the residues are chemically modified, for example, with 2′ substituents such as O-Methyl and Fluoro.[18] Recently a clinical trial has begun of a ribozyme targeted on HIV.[19]

Methods of delivery

Apart from direct injection of oligomers, several methods of delivery have been tried *in vitro*, by forming complexes with positively charged lipids,[20] by incorporation into liposomes,[5] and by electroporation. At present no single method has been reported that combines the properties of both efficacy and targeting. Two parameters may be helpful, (a) the fact that oligomers tend to accumulate in specific tissues,[8] thus providing a crude tissue targeting, and (b) the use of sterically hindered immunoliposomes used to deliver genes in gene therapy, may also become carriers for oligomers. It should be noted that oligomers are almost certainly protein-bound *in vivo*, and the impact of this fact on their efficacy, delivery, and excretion has not been clarified. Specific examples of systemic delivery to block or promote repression are discussed in comparison with the results for the corresponding stably produced antisense RNA in transfected clonal lines in the next section.

Clinical trials

The ability to carry out clinical trials of oligonucleotides represents the accumulation of several years of effort, not only preparing oligodeoxynucleotides and analogs in large quantities,[21] but also raising this synthesis to the level of good manufacturing practice. As an example of the use of an antisense oligonucleotide in an animal disease model, an anti-c-myb phosphorothioate oligonucleotide has been reported to result in the elongation of life of *nu/nu* athymic mice in a model tumor system.[22] Clinical trials of antisense oligonucleotides are underway against five diseases (Table 1).[22–26] Overall several

APPENDIX B

CLINICAL FRONTIERS

EXHIBIT 2

Antisense strategies and therapeutic applications

DAVID A. PUTNAM

Abstract: The concepts underlying the antisense approach to disease therapy are discussed, and potential applications are examined.

Antisense therapeutic agents bind to DNA or RNA sequences, blocking the synthesis of cellular proteins with unparalleled specificity. Transcription and translation are the two processes with which the agents interfere. There are three major classes of antisense agents: antisense sequences, commonly called antisense oligonucleotides; antigene sequences; and ribozymes. Antisense sequences are derivatives of nucleic acids that hybridize cytosolic messenger RNA (mRNA) sense strands through hydrogen bonding to complementary nucleic acid bases. Antigene sequences hybridize double-stranded DNA in the nucleus, forming triple helixes. Ribozymes, rather than inhibiting protein synthesis simply by binding to a single targeted mRNA, combine enzymatic processes with the specificity of antisense base pairing, creating a molecule that can incapacitate multiple targeted mRNAs. Antisense therapeutic agents are being investigated in vitro and in vivo for use in treating human immunodeficiency virus infection, hepatitis B virus infection, herpes simplex virus infection, papillomavirus infection, cancer, restenosis, rheumatoid arthritis, and allergic disorders. Although many results are preliminary, some are promising and have led to clinical trials. A major goal in developing methods of delivering antisense agents is to reduce their susceptibility to nucleases while retaining their ability to bind to targeted sites. Modification of the phosphodiester linkages in oligonucleotides can lend the sequences enzymatic stability without affecting their binding capacities. Carrier systems designed to protect the antisense structure and improve passage through the cell membrane include liposomes, water-soluble polymers, and nanoparticles. The pharmacokinetics of antisense agents are under investigation.

Antisense therapeutic agents have the potential to become an integral part of medicinal regimens.

Index terms: Allergies; Arthritis; Gene therapy; Genetic engineering; Hepatitis B infections; Herpes simplex infections; HIV infections; Neoplasms; Oligonucleotides; Papillomavirus infections; Restenosis

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One way drugs act is by inhibiting the function of a protein or enzyme by binding to and inactivating its active site. For example, antineoplastic agents like fluorouracil inhibit the activity of key cellular proteins in an attempt to kill cancer cells, and antiviral compounds like zidovudine inhibit the activity of key viral replication proteins in an attempt to hinder viral reproduction. Such mechanisms of action generally produce undesirable effects and lack specificity, limiting the efficacy of therapy. A more rational approach is to inhibit the production of the protein altogether. That is precisely the approach of antisense therapy. Rather than inhibiting the metabolic action of a protein, antisense therapeutic agents inhibit the synthesis of a specific protein by blocking the intracellular mechanisms involved in its production. This mechanism has a specificity unattainable with currently available therapies.

Recent advancements in biotechnology have brought the antisense approach to the forefront of therapeutic research. The purpose of this article is to acquaint the reader with antisense therapeutic strate-

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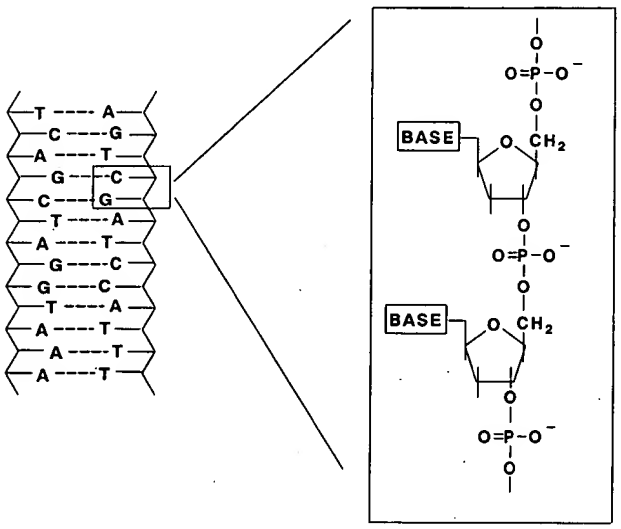
Figure 1. Left panel: Duplex strand of DNA. The dashed lines between the bases (adenine [A], thymine [T], cytosine [C], and guanine [G]) represent hydrogen bonds. Right panel: Chemical structure of the DNA-strand backbone (deoxyribose and phosphate groups).



gies and their potential applications.

General function of cellular genes

Genes consist of two side-by-side chains of deoxyribonucleotides held together in a double helix by base pairs. There are four deoxyribonucleotide bases: adenine (A), cytosine (C), guanine (G), and thymine (T). These bases are linked between strands by hydrogen bonds (Figure 1). The base A pairs with the base T, and the base G pairs with the base C. Transcription (also termed gene expression) involves the synthesis of messenger RNA (mRNA) sequences using the DNA base sequence as a template. RNA is structurally similar to DNA except for a modification in the ribose carbohydrate (DNA contains deoxyribose, RNA contains ribose) and substitution of uracil (U) bases for the T bases. During transcription, a template A base encodes for a U mRNA base, a template G base encodes for a C mRNA base, a template C base encodes for a G mRNA base, and a template T base encodes for an A mRNA base.

After transcription, mRNA may undergo extensive alteration (RNA processing) before the final mRNA is formed. The final mRNA chain is transferred from the nucleus to the cytoplasm, where it combines with a ribosome. The mRNA sequence then encodes for the protein primary sequence (i.e., the sequence of the amino acids). The process of decoding the mRNA sequence to form a protein is called translation. A series of three mRNA nucleotides, known as a codon, directs the addition of a specific amino acid to the growing protein chain.

Principles of antisense therapeutics

Transcription and translation are the two processes that antisense therapeutic agents interfere with to inhibit the synthesis of proteins. The three major subclasses of antisense therapeutic agents (antisense sequences, antigene sequences, and ribozymes) are defined according to their mechanisms of action. Each of these classes is described below, and the mechanisms of action are diagrammed in Figure 2.

Antisense sequences. Antisense sequences are derivatives of nucleic acids that bind to (hybridize) cytosolic mRNA sense strands through hydrogen bonding to complementary nucleic acid bases. Antisense sequences typically have short nucleic acid sequences and are therefore commonly termed antisense oligonucleotides.

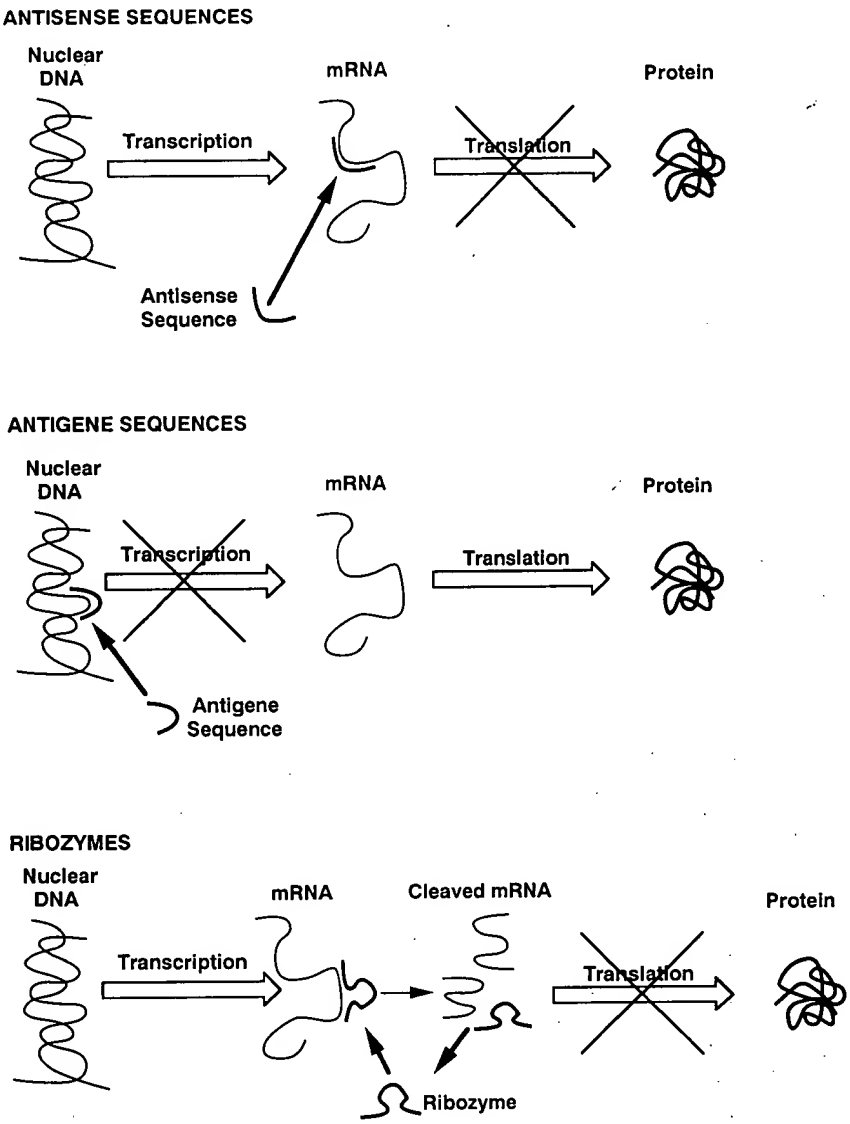
The human genome contains some 3 billion nucleic acids arranged in a relatively specific sequence. Because of the immense number of nucleic acids, it is statistically unlikely that a specific sequence of 17 or more nucleotides will occur more than once.[1] Therefore, antisense oligonucleotides are usually at least 17 nucleic acids long (i.e., a 17-mer oligonucleotide). The longer the sequence, the greater the affinity of the antisense strand for its targeted sequence. However, extremely long antisense oligonucleotides are not practical, since the cost of their synthesis and their decreased cellular uptake become counterproductive.

Cellular synthesis of a protein requires the transfer of information from nuclear DNA to cytosolic ribosomes via mRNA, termed the sense mRNA. The sense mRNA is a single strand of nucleic acids. For a protein to be synthesized, the mRNA must remain single-stranded to be translated by the ribosomal units. Antisense sequences are designed to recognize and combine with specific nucleic acid sequences contained within an mRNA, forming a double-stranded hybrid that cannot be translated into the encoded protein. Translation is blocked by inhibition of translation initiation[2] or by formation of a double strand that is a substrate for ribonuclease (RNase) H (an enzyme that cleaves duplex RNA strands).[3] Consequently, the synthesis of the protein is inhibited.

Antigene sequences. Just as antisense sequences hybridize cytosolic mRNA to inhibit protein synthesis, antigene sequences hybridize double-stranded DNA in the cell nucleus, forming triple helixes (three-stranded). This hybridization results in inhibition of transcription either by inhibiting the binding of sequence-specific DNA proteins required for the transcriptional process or by directly preventing the transcription to mRNA.[4]

At present, antigene sequences can bind only to a limited number of DNA duplex strands and do not have the sequence-specific potential of the antisense oligonucleotides. This limited potential is due to the fact that the antigene sequences do not bind to the targeted DNA via base pairing of the type shown in Figure 1. Rather, the antigene sequences bind to the duplex DNA to form a triple helix through what are

Figure 2. Mechanisms of action of the three antisense therapeutic subclasses. Antisense sequences recognize and bind to mRNA to inhibit protein synthesis. Antigene sequences recognize and bind to nuclear DNA to inhibit mRNA synthesis and subsequently inhibit protein synthesis. Ribozymes recognize and bind to mRNA and catalyze its cleavage, inhibiting protein synthesis.



called Hoogsteen base pairs. Hoogsteen base pairing occurs predominantly at duplex DNA sites that are rich in A–T bonds or in G–C bonds.[5,6] Since this type of sequence does not lend itself to sequence-specific binding, the specificity of the antigene sequence can be compromised, resulting in decreased therapeutic potential.

Ribozymes. In addition to carrying genetic information, RNA can enzymatically catalyze the cleavage of specific mRNA sequences. These catalytic RNAs are termed ribozymes (not to be confused with ribosomes, the cellular organelles). Rather than inhibiting protein synthesis simply by binding to a single targeted mRNA, ribozymes combine enzymatic processes with the specificity of antisense base pairing, creating a molecule capable of incapacitating numerous targeted mRNAs.[7] The ribozyme structure is not changed after the cleavage process, so ribozymes are true catalysts.

Four main types of ribozymes have been reported: tetrahymena group I, RNase P, hammerhead, and hairpin.[8] The ribozymes are classified according to their biological origin and their catalytic motif (the three-dimensional structure of the catalytic section of the molecule). The three-dimensional structure of ribozymes is very important to their function; disruption of the catalytic motif can easily render a ribozyme inactive. Ribozymes are naturally occurring and therefore have natural substrates. However, ribozymes can be adapted to cleave other substrates (such as targeted mRNAs) as well by the modification of specific "guide sequences" that can be designed into the ribozyme to direct it to a specific targeted mRNA.

Of the four major types of ribozymes reported, the hammerhead is the simplest and the most highly studied. Its guide sequence consists of flanking regions that bind to the targeted mRNA and bring the catalytic center into position to facilitate cleavage. Hammerhead ribozymes are also readily synthesized by chemical,[9] biochemical,[10] and biological[11] methods. For these reasons, the hammerhead is considered the ribozyme with the highest therapeutic potential.

Potential therapeutic applications

The potential therapeutic applications of antisense therapeutic agents are far-reaching. In theory, any disease state with a gene-expression component has the potential to be treated with antisense agents. To date, viral infections and cancer have been the primary disease states under investigation for treatment with these agents; however, other, less obvious potential uses, such as for restenosis, rheumatoid arthritis, and allergies, have also been studied.

Viral infections. The genetic makeup and life cycle of viruses are very different from those of human cells. These differences provide an excellent opportunity for the successful application of antisense therapeutics. Perhaps the most important potential antiviral therapeutic application is in human immunodeficiency virus (HIV) infection.

HIV infection. The first report on the possibility of using antisense agents against HIV was published in 1986.[12] Antisense oligonucleotides designed to recognize a vital HIV gene (*tat*) were incubated with cultured lymphocytes and human peripheral blood cells. The results showed there was anti-HIV activity, determined by the inhibition of HIV reverse-transcriptase activity, relative to control cells; however, the study did not control for possible sequence-nonspecific effects (antiviral activity due to the inhibition of sequences other than the targeted sequence). Additional experiments also showed marked sequence-nonspecific effects of the agents.[13-15] One factor influencing these results was the type of anti-HIV assay used. The investigators used a viral challenge (de novo) assay that can be influenced by interruption of the viral life cycle at any point.[16] An improved assay with chronically infected cells showed that antisense oligonucleotides were indeed able to inhibit HIV expression in a sequence-specific manner.[17]

Ribozymes also show promise for anti-HIV therapy. In a 1990 paper, Chang et al.[18] reported activity of the hammerhead ribozyme against HIV. The hairpin ribozyme also has anti-HIV activity but is less promising than the hammerhead type because of its complex catalytic center.[19]

Hepatitis B virus infection. Interferon alfa is widely used to treat chronic hepatitis type B, but it is effective in only about a third of patients.[20] The ability of antisense oligonucleotides to inhibit hepatitis B virus gene expression has been demonstrated both in vitro and in vivo. Each model involves duck hepatitis B virus, which is closely related to human hepatitis B virus.[21] Two types of antisense oligonucleotides showed the ability to inhibit duck hepatitis B virus gene expression.[22,23] One type was incubated directly with primary duck hepatocyte cultures and demonstrated the effect of sequence specificity: Nine oligomers were tested, and two showed exceptionally high efficiency owing to the particular section of the hepatitis B virus genome they recognized.[22] The other type of antisense oligonucleotide was directed at a specific liver cell surface receptor.[23] The targeted antisense oligonucleotide was taken up by cultured human hepatoma cells 12 times faster than a nontargeted oligonucleotide. Uptake of the targeted sequence by the hepatoma cells was susceptible to competitive inhibition by specific added compounds, strongly suggesting a cellular uptake mechanism of receptor-mediated endocytosis.

When day-old Pekin ducklings were infected with duck hepatitis B virus and then, two weeks later, given daily intravenous injections of an antisense oligonucleotide for 10 days, there was reproducible dose-dependent inhibition of viral replication.[22] However, persistent precursors of viral transcription were found in the duck liver cell nuclei; these precursors can result in reactivation of viral replication after the end of therapy. Extended treatment was suggested to eliminate this problem.

Herpes simplex virus infection. Herpes simplex virus type 1 (HSV-1) is potentially amenable to therapy with antisense oligonucleotides. In vitro treatment of monkey kidney cells with an oligonucleotide complementary to the acceptor splice junction of HSV-1 immediate early pre-mRNAs 4 and 5 (viral gene sequences that regulate the early growth of the virus) caused a dose-dependent decrease in HSV-1 titers.[24] While the exact function of the products of HSV-1 immediate early pre-mRNAs 4 and 5 is unknown, it is known that their genes have a regulatory role in the early stages of the replication of this virus; this may increase the oligonucleotide's therapeutic activity. Up to 98% inhibition of viral growth was obtained with exposure to 100 μM of the oligonucleotide.

Epstein-Barr virus infection. An extension of the use of antisense sequences against herpesviruses is the use of antisense oligomers to treat latent Epstein-Barr virus (EBV) infection. Pagano et al.[25] tested two types of antisense oligonucleotides recognizing various coding regions in the EBV genome. The investigators reported that the unmodified oligomer caused a 90% reduction in the number of viral episomes present in latently EBV-infected Burkitt's lymphoma cells in vitro. However, the result could not be consistently reproduced. The authors attributed the irreproducibility to cell division problems. For example, cells successfully cleared of the viral episome may not grow as readily as infected cells, skewing the results. Although the results were not

consistent, episome inhibition was dose dependent, suggesting the potential for successful application of this treatment.

Papillomavirus infection. Antisense oligonucleotides complementary to specific gene sequences of human papillomavirus have shown potential for use against genital warts. E2, a papillomavirus gene sequence that encodes for E2 proteins critical for viral transcription and viral DNA replication, was targeted with antisense oligomers, one of which, ISIS 2105, showed particular activity by inhibiting up to 70% of the E2 function.[26] The oligonucleotide is in clinical trials for the treatment of genital warts.

Cancer. Because cancer cells arise from normal cells, their biochemistries do not differ greatly. To effectively kill cancer cells while sparing normal cells, vital processes or regulators of vital processes occurring in cancer cells but absent in noncancerous cells must be found and exploited. More likely, however, is the identification of processes on which cancer cells are more dependent for proliferation than noncancerous cells. Antisense therapeutic agents have excellent potential to block the processes responsible for malignant cell growth. Proto-oncogenes are the malignant elements that antisense therapeutic agents have the greatest potential to inhibit.

Proto-oncogenes are naturally occurring gene sequences in the human genome that are implicated in certain malignancies. They have the potential to encode for or regulate genes that encode for products that can transform cells into malignant cells. Proto-oncogenes play a role in the normal growth and differentiation of cells but are overexpressed in the malignant state, which results in continued cell division and poor cell differentiation. Theoretically, if overexpression of a proto-oncogene responsible for a malignancy can be halted or down-regulated, there should be a reversal of the malignant state.

Various proto-oncogenes have been targeted by researchers using antisense agents. The main proto-oncogenes studied to date are the *myc* family (including c-*myc* and N-*myc*), c-*myb*, c-*fos*, the *ras* family (including N-*ras* and c-H-*ras*), BCL-2, c-*raf*-1, *cdc*-2, and c-*mos*. Thorough discussions of these proto-oncogenes and their targeting are available elsewhere.[27-30]

Studies of the use of antisense techniques against proto-oncogenes have met with various degrees of success. The leukemia cell line HL-60 overexpresses c-*myc* and is a good model for studying the effect of c-*myc* on malignant cell growth. Incubation of an anti-c-*myc* 15-mer with HL-60 cells resulted in a sequence-specific inhibition of c-*myc* expression.[31] In another study in the same cell line, an antisense oligonucleotide complementary to c-*myc* induced apoptosis of the malignant cells.[32] An interesting result of these experiments is that while inhibition of c-*myc* resulting from exposure to the antisense oligonucleotide induced apoptosis, removal of the oligonucleotide (resulting in c-*myc* up-regulation) induced further apoptosis. The clinical relevance of this finding is that leukemia cells may be potentially suppressed by alternating therapy between c-*myc* suppression and c-*myc* induction.

McManaway et al.[33] found that the growth of Burkitt's lymphoma cells that expressed c-*myc* because of a deviant promoter sequence was specifically inhibited by an antisense 21-mer. The study showed the remarkable specificity of antisense agents, since only Burkitt's lymphoma cells with the abnormal promoter were inhibited. N-*myc* is not as prevalent an oncogene as c-*myc*; however, its expression is closely associated with cell differentiation. The growth rate of a neuroepithelioma cell line (CHP100) that expressed N-*myc* was significantly reduced by an antisense 15-mer.[34] In addition, the growth rate of the same cell line was slowed by an episomal vector coding for the intracellular synthesis of an anti-N-*myc* antisense sequence.[35] While growth rates were slowed in these studies, the tumorigenicity of the cell lines remained intact. This demonstrates that the ability of antisense therapeutic agents to eliminate cancer cells is strongly dependent on the targeted sequence.

C-*myb* may be linked to the regulation of cell growth, particularly the growth of malignant hematopoietic cells like T-cell leukemias and myeloid leukemias. Sequence-specific inhibition of the growth of cultured leukemia cell lines and of cells isolated from patients was achieved with an 18-mer designed to be complementary to c-*myb* mRNA.[36,37] The down-regulation of c-*myb* expression in the cultured cell lines was followed by reduced growth of the cells; however, the growth of about 25% of the leukemia cells isolated from patients was not affected by the antisense sequence complementary to c-*myb*. It was postulated that in some cases leukemia cells can acquire the genetic characteristics necessary to nullify the need for c-*myb* expression for malignant growth.

Another approach to treating leukemia, or more specifically chronic myelogenous leukemia (CML), with antisense oligonucleotides is to use them to purge the bone marrow of CML patients undergoing autologous bone marrow transplantation. Gewirtz[38] studied an antisense oligonucleotide complementary to c-*myb* to determine if the oligonucleotide could eliminate malignant CML cells from the bone marrow and to determine if it could eliminate the malignant CML cells from the marrow autograft while leaving enough normal cells to permit successful transplantation. The antisense oligonucleotide seemed to perform both functions, and the antisense oligonucleotide is now in clinical trials.[39]

Restenosis. Restenosis resulting from neointimal hyperplasia after coronary angioplasty occurs in 25–50% of patients within six months of the procedure.[40] Therapy with traditional medicines, such as

heparin, interferon gamma, and angiotensin-converting-enzyme inhibitors, has failed to significantly reduce the occurrence of restenosis in humans.[41] The cause of restenosis after angioplasty is thought to be overaggressive healing in which the vascular damage incurred during the procedure causes excessive proliferation of the smooth muscle cells. The proliferating smooth muscle cells secrete extracellular matrix, and narrowing of the vessel lumen and restriction of blood flow result.[40] Antisense agents provide a means of specifically inhibiting the proliferation of smooth muscle cells after vascular injury and are promising agents for the prevention of restenosis.

In vitro, antisense oligonucleotides targeted against the encoding genes for proliferating cell nuclear antigen,[42,43] c-*myc*,[44,45] and c-*myb*[46] have the ability to suppress smooth muscle cell growth. In vivo studies in rats have also yielded promising results. Antisense oligonucleotides recognizing c-*myc*,[47] cdk 2,[48] and cdc 2[48] inhibited the proliferation of smooth muscle cells after carotid artery angioplasty. Individual antisense oligonucleotides could not completely inhibit neointimal formation, but a combination of antisense cdc 2 and cdk 2 oligonucleotides inhibited nearly all neointimal formation.

Rheumatoid arthritis. Chondrocytes in articular cartilage produce proteoglycans to promote the tissue's structural integrity. Interleukin-1 induces chondrocytes to synthesize interleukin-6. The result is inhibition of proteoglycan synthesis.[49] This process is thought to play an important role in the cartilage destruction associated with rheumatoid arthritis.

Cultured human articular cartilage was used to study the ability of an antisense 18-mer (directed against the mRNA encoding for interleukin-6) to influence the interleukin-1-induced inhibition of proteoglycan synthesis.[50] The oligonucleotide prevented the interleukin-1-induced production of interleukin-6 in the cultured cartilage and the inhibition of proteoglycan synthesis. This selective inhibition of cytokine production suggests that antisense agents may be of value in other inflammatory disease states.

Allergic disorders. Production of immunoglobulin E (IgE) antibodies to environmental allergens occurs during the typical allergic reaction. In an attempt to reduce the formation of IgE and thus the severity of an allergic reaction, Hall and Brostoff[51] studied an antisense oligonucleotide complementary to an IgE-encoding gene sequence in vitro. The 12-mer reduced IgE production in a dose-dependent manner and had no effect on the production of immunoglobulins G and M. Although these results are preliminary, the therapeutic potential of these IgE-reducing agents is high. They might be easily administered intranasally to treat allergic rhinitis.

Delivery of antisense therapeutic agents

Oligonucleotide modifications. The major concern in the delivery of antisense agents to cells is the susceptibility of the agents to degradation by enzymes in the blood and the cells. Nucleases in blood and cells rapidly degrade naturally occurring oligonucleotides.[52,53] Therefore, the major goal in developing methods of delivery is to reduce the susceptibility of antisense agents to nucleases while retaining their ability to bind to targeted sites.

The phosphodiester linkages in naturally occurring oligonucleotides are the targets of nuclease activity. Chemical modification of these linkages can give the oligonucleotides enzymatic stability without affecting their binding capacities. The major types of modifications are shown in Figure 3. The modified linkages are expressly designed to retain the spatial configurations of the naturally occurring phosphodiester bonds while resisting the catalytic activity of the nucleases.

Of the modified linkages shown in Figure 3, the phosphorothioates are the most studied and most promising.[54-56] These modified antisense oligonucleotides retain their affinity for targeted sites and have greater resistance to the catalytic activity of nucleases. Large-scale, economical synthesis of these modified sequences has been reported.[57] Automated DNA synthesizers can generate a wide variety of modified sequences.

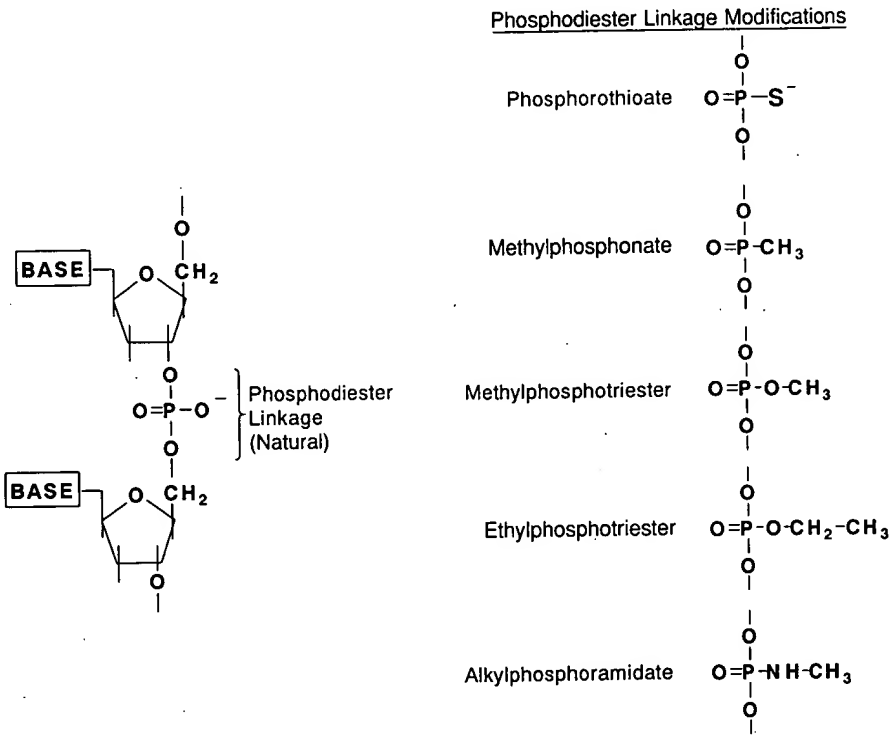
Since the three-dimensional structure of ribozymes is important to their enzymatic activity, chemical substitution of their natural phosphodiester linkages with bonds like those shown in Figure 3 can greatly decrease their catalytic activity. Linkages such as phosphorothioates or methylphosphonates can alter the spatial orientation of the ribozyme structure and greatly influence ribozyme antisense activity. Specific chimeric (containing both RNA- and DNA-type structures) RNase-resistant ribozymes have therefore been synthesized.[58] These chimeric ribozymes retain their site-specific cleavage activity.

Modification of the sugar moiety (ribose) of antisense oligonucleotides may enhance their binding affinity while providing resistance to nucleases.[59] Oligonucleotides containing modifications of the base moiety also have potential therapeutic applications. For example, sequences in which uracil or cytosine is replaced by a propyne group at the C-5 position show high affinity for RNA targets.[60]

Carrier systems. Another factor influencing the delivery of antisense therapeutic agents is the low permeability of cell membranes to these compounds. Since their sites of activity are in the cytoplasm or nucleus, penetration of the agent into the cell is essential. Although antisense sequences are internalized by cells,[61,62] the extent of uptake may not be adequate for sustained activity in vivo. Therefore, carrier systems have been designed to protect the antisense structure against degradation and to improve passage through the cell membrane.

The liposome is a well-understood system of drug

Figure 3. Major types of phosphodiester linkage modifications used for antisense therapeutic agents.



delivery and offers characteristics that favor its use for delivering antisense agents. Liposomes are spherical structures consisting of multiple phospholipid bilayers arranged to create hydrophobic bands alternating with hydrophilic bands (multilamellar) or consisting of a single phospholipid bilayer encasing an aqueous core (unilamellar). Drugs, in this case antisense sequences, can either be partitioned into the hydrophobic layers or be dissolved in the aqueous layers. The drug is released by partitioning back out of the liposome or when the liposome structure is disrupted. The composition, type, and size of liposomes can be altered to impart different characteristics.

A drawback of liposomes is their relatively short circulation time. Liposomes are rapidly recognized by the reticuloendothelial system (RES)—typically by monocytes and macrophages in the liver and spleen—and taken up by endocytosis and deactivated. If the targeted site is within these RES cells, then liposomes are excellent delivery systems; however, the targets are often elsewhere in the body. Advances in liposome technology have created liposomes whose circulation times have been increased by minimizing their recognition by the RES. For example, polyethylene glycol added to the surfaces of liposomes repels proteins involved in their recognition and therefore increases their circulation time.[63,64] Polyethylene glycol-modified liposomes are also known as Stealth liposomes. Adding targeting moieties, such as monoclonal antibodies, to long-circulating liposomes can guide the antisense sequence to the desired subset of cells.[65]

A liposome-incorporated antisense methylphosphonate oligonucleotide that is cytotoxic to CML cells inhibited the growth of CML cell lines in vitro in a dose-dependent manner.[66] In addition to being an effective carrier of the antisense oligonucleotide, the liposome served as a solubilizer for the water-insoluble drug. In another study, lipofectin, a cationic lipid, enhanced the delivery of large antisense therapeutic agents (ribozymes directed against MRNAs encoding for human urokinase receptors) to the cytoplasm of cultured osteosarcoma cells.[67] The ribozymes showed increased stability to enzymatic degradation.

Another approach is the use of water-soluble polymeric carriers, such as poly-L-lysine.[68] Poly-L-lysine maintains a highly positive net charge at physiological pH and thus is electrostatically attracted to the negatively charged cell membrane; the result is nonspecific adsorptive endocytosis.[69] Chemically attaching an antisense therapeutic agent to poly-L-lysine may increase the concentration of the agent at the cell surface and thus the potential for endocytotic uptake.[70] It is postulated that the antisense therapeutic agent is released from the poly-L-lysine after lysosomal proteolysis of the carrier.[68]

Synthetic water-soluble polymeric carriers may be of use as well, particularly for the treatment of cancer.[71] These carriers can be tailor-made to release specific therapeutic agents within targeted cells.[72]

Nanoparticles have been used to deliver these com-

pounds. Antisense oligonucleotides were adsorbed onto polyalkylcyanoacrylate-based nanoparticles and studied for their ability to inhibit the growth of a tumorigenic human mammary cell line.[73] The nanoparticle-adsorbed oligonucleotides were 100 times more active in inhibiting cell growth than free oligonucleotides.

Pharmacokinetics

To date, the pharmacokinetics of only antisense oligonucleotides have been studied in detail. The pharmacokinetic profile of antisense sequences depends primarily on the type of bond linking the bases.

Antisense oligonucleotides with phosphodiester linkages have very short half-lives. For example, an antisense 25-mer injected intravenously into monkeys had a half-life of only five minutes.[74] Such a short half-life is one reason for the poor therapeutic potential of sequences with phosphodiester linkages.

Methylphosphonate-based antisense oligonucleotides have a longer half-life than the phosphodiester-based sequences. An antisense 12-mer injected intravenously into mice had an elimination half-life of 17 minutes.[75] In addition, the authors reported a time to peak tissue concentration of five minutes, with the highest concentration occurring in renal tissue. Of the total dose, 70% was eliminated in the urine between 60 and 120 minutes after administration.

Antisense oligonucleotides with phosphorothioate linkages have perhaps the most favorable pharmacokinetic profile. The pharmacokinetics of these agents have been reported for mice, rats, monkeys, and humans.[74] Values after i.v. administration are similar for mice and rats. Both species show a biphasic plasma elimination profile, with similar distribution-phase half-lives (mice, 0.53 hours; rats, 0.83–1.11 hours) and elimination-phase half-lives (40 and 33.45–35.15 hours). The liver and kidneys had the highest uptake of oligonucleotides. The oligonucleotides were primarily excreted in the urine for both species, with about 30% being eliminated within the first 24 hours.

Similar results were seen after single i.v. injections of a phosphorothioate-based oligonucleotide into monkeys. The plasma concentrations again showed a biphasic elimination profile, with a distribution-phase half-life of 0.6–1 hour and an elimination-phase half-life of 42.2–56.3 hours. When a 25-mer phosphorothioate-based oligonucleotide was administered by two-hour i.v. infusion into six HIV-infected humans, the mean distribution-phase half-life was 0.18 hour, and the mean elimination-phase half-life was 26.71 hours. Elimination occurred primarily through the urine; a mean of 49% of the dose was eliminated (as degraded oligonucleotide) after 24 hours and a mean of 70% (as degraded oligonucleotide) after 96 hours.

In another study, a phosphorothioate-based oligonucleotide was administered by continuous i.v. infusion over 10 days to five patients with acute myelogenous leukemia or myelodysplastic syndrome.[76] Between 30% and 62% of the total dose of the 20-mer was eliminated in the urine. Only 9–18% of the total dose detected in the urine was unmetabolized oligonucleotide. The estimated elimination half-life ranged from 4.9 to 14.7 days. The authors attributed the wide range to the heterogeneity of the patients.

Conclusion

Antisense therapeutic agents—antisense sequences, antigene sequences, and ribozymes—have the potential to become an integral part of medicinal regimens. They offer greater treatment specificity because they block the synthesis of a specific protein rather than inhibiting the metabolic effects of that protein. Antisense therapeutic agents must be designed so they are resistant to enzymatic degradation while retaining affinity for their targeted sequences. Carrier systems can protect the antisense compounds from nucleases and deliver the agents to specific cells. The therapeutic potential of antisense compounds spans a wide range of diseases, notably HIV infection and cancer. Intensive research in human genetics will certainly lead to more therapeutic applications.

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APPENDIX C

Advances in antisense efficacy and delivery

Meeting report

Antisense drug therapy applies the techniques of rational drug design to the development of a range of drugs each of which targets a well-characterized gene product. Antisense oligonucleotides are relatively easy to design and to synthesize, and have a predictable high affinity for the disease-targeted gene, leading to a minimization of side effects. However, the development of antisense-oligonucleotide therapy has not been as simple as was first believed, and many critical issues have been highlighted. These include concerns about oligonucleotide modifications, cellular uptake and targeted delivery, sequence-specific and non-sequence-specific biological effects, pharmacokinetics and pharmacodynamics. These issues were addressed by 13 speakers from academic and biotechnology laboratories at a recent meeting*.

Phosphorothioate oligodeoxynucleotides

The phosphorothioate oligonucleotides (PS-oligonucleotides), in which one of the non-bridged oxygens of the internucleotide linkage has been replaced with sulfur, are among the most studied oligonucleotide analogs. PS-oligonucleotides are resistant to nucleases, and show sequence-specific and non-sequence-specific biological activity in various assay systems. Specific PS-oligonucleotides have been shown to be active against human papilloma virus (HPV), human cytomegalovirus (HCMV), intercellular adhesion molecule 1 (ICAM-1), Ki-*ras*, and protein kinase C (David Ecker, ISIS Pharmaceuticals, Carlsbad, CA, USA), human immunodeficiency virus type 1 (HIV-1), influenza virus (Sudhir Agrawal, Hybridon, Worcester, MA, USA), *p53* (Patrick Iverson, University of Nebraska Medical Center, Omaha, NE, USA), C-*myb* (Mariusz Ratajczak, University of Pennsylvania School of Medicine, Philadelphia, PA, USA), and *relA* (Ramaswamy Narayanan, Hoffmann La Roche, Nutley, NJ, USA). The activity of specific PS-oligonucleotides for the above targets was in the range of 10 nM to 5 μM, Generally, at low doses, PS-oligonucleotides showed sequence-specific effects, but at higher concentrations, non-sequence-specific effects were observed. Oligonucleotides containing four contiguous guanines showed unusual non-sequence-specific effects (Narayanan).

Cellular uptake and targeted delivery

It is generally agreed that oligonucleotides enter cells by some form of endocytosis. As it is not possible to achieve high intracellular concentrations by this route, several groups have attempted to develop methods to improve the cellular uptake of oligonucleotides. When conjugated with polyaminolipids, such as spermidine-cholesterol or spermine-cholesterol, oligonucleotides showed significantly improved cellular uptake compared with oligonucleotides alone, or oligonucleotides delivered with Lipofectin™ (Nilabh Choudhary, Triplex Pharmaceutical Corporation, The Woodlands, TX, USA). Although the exact uptake mechanism has not yet been defined, fluorescently labeled oligonucleotide-polyaminolipid conjugates showed enhanced nuclear localization. This suggests that these complexes may be suitable for delivering triplex oligonucleotides, which must reach the nucleus for therapeutic activity.

The issue of targeting oligonucleotides to specific cells or tissues was addressed by Annie-Claude Roche (CBM CNRS, Orleans, France) and Saghir Akhtar (Aston University, Birmingham, UK). Roche's group has been targeting oligonucleotides to specific cells via specific sugar-binding receptors (membrane lectins) that are found on the surface of macrophages (mannose lectin), and on the surface of hepatocytes (galactose lectin). The desired sugar residues were attached to a protein carrier (e.g. bovine serum albumin) and then conjugated with oligonucleotides directed against HIV or H-*ras*. The lectin-conjugated oligonucleotides showed improved cellular uptake and *in vitro* activity in cell lines bearing the targeted lectins.

Akhtar's group has been attempting to deliver oligonucleotides to the brain. Oligonucleotides are unable to cross the blood–brain barrier (BBB), mainly because of the presence of tight intercellular junctions and low pinocytic activity in brain endothelial cells. Iron is readily transported into the brain by transferrin receptors, which are situated in abundance at the BBB. Taking advantage of this transport mechanism, Akhtar's group has conjugated oligonucleotides with monoclonal antibodies raised against the transferrin receptor. The monoclonal antibodies bind to the transferrin receptor and should cross the BBB without degradation, presumably also carrying the oligonucleotide across. Although preliminary data suggest that this may be possible *in vitro*, it remains to be seen whether this technique will improve the pharmacokinetic profile of oligonucleotides *in vivo*.

Intracellular trafficking and localization

The intracellular trafficking and distribution of oligonucleotides determines the extent to which an 'internalized' oligonucleotide is available to interact directly with its biological targets. Oligonucleotides introduced directly into the cytosol migrate rapidly into the nucleus, but oligonucleotides and their conjugates that enter cells by endocytic pathways initially localize within endosomal and/or lysosomal vesicles. These oligonucleotides must first be released from these acidic compartments in order to become bioavailable.

Efflux (exocytosis) kinetics of oligonucleotides in HL-60 cells suggest that the intracellular trafficking of phosphodiesters and PS-oligonucleotides is different. Upon internalization, phosphodiesters predominantly (65%) reside in shallow compartments, from which efflux is relatively rapid, whereas PS-oligonucleotides largely (80%) reside in deeper compartments, from which efflux appears to be slower (Cy Stein, Columbia University College of

*The meeting 'Antisense Therapy, Efficacy and Delivery of Antisense and Ribozyme Oligonucleotides', organized by the IBC, was held at Charing Cross Medical School, London, UK, 23–24 February 1995.

Physicians and Surgeons, New York, NY, USA). To prevent oligonucleotides becoming trapped within these compartments, two delivery strategies are being investigated (Roche). The first strategy makes use of peptide–oligonucleotide conjugates that have an endoplasmic reticulum-seeking KDEL amino acid residue motif. The aim is to force oligonucleotides to exit from endosomal or lysosomal compartments, or both, more rapidly. The second strategy is to use peptides derived from the influenza virus hemagglutinin HA2 protein that have been linked to glycosylated polylysine carriers. This has resulted in the improved activity of anti-*gag* oligonucleotides in HIV-infected cell lines. The use of pH-sensitive liposomes to destabilize endosomal/lysosomal membranes, thus enhancing the biological activity of oligonucleotides targeted to the Friend retrovirus, was also discussed (Claude Malvy, Institut Gustave-Roussy, Villejuif, France).

Sustained delivery systems

For many *in vivo* applications, oligonucleotides must be administered repeatedly, in order to obtain the desired biological effects, partly because oligonucleotides are rapidly eliminated, and also because the target gene products have long half-lives. Biodegradable polymer matrices that can be injected or implanted *in vivo* may provide a delivery system that not only protects oligonucleotides from nuclease digestion, but also provides sustained delivery over a period of up to several months (Akhtar). Such delivery devices degrade to non-toxic metabolites and, therefore, do not require surgical removal from the body. The use of these delivery systems may also decrease the non-specific effects of oligonucleotides because low doses are delivered directly to the desired site of action.

Pharmacokinetics of PS-oligonucleotides

The pharmacokinetics of PS-oligonucleotides of varying length and base composition have been studied in mice (Agrawal), rats (Ecker, Iverson and Agrawal) and monkeys (Iverson). Pharmacokinetic studies carried out with ^{14}C-labeled oligonucleotides (Ecker) have demonstrated that PS-oligonucleotides have a long plasma half-life and are distributed to most tissues, except the brain; the major route of elimination from the body is in expired air. Pharmacokinetic studies carried out with ^{35}S-labeled PS-oligonucleotides showed a very similar profile of plasma clearance and tissue distribution, but showed that PS-oligonucleotides are eliminated mainly in urine (Agrawal and Iverson). PS-oligonucleotide metabolism occurs in various tissues, with degradation occurring primarily from the 3′-end.

Safety of PS-oligonucleotides

The toxicological properties of PS-oligonucleotides of varying length and base composition have been studied; PS-oligonucleotides are tolerated well in mice and rats. However, in monkeys, dose-limiting toxicities are related to complement activation (Agrawal) and/or thrombin binding (Ecker).

Human clinical trials

Human clinical trials are under way using PS-oligonucleotides for HPV, HCMV and ICAM-1 (Ecker), HIV-1 (Agrawal), *p53* (Iverson), and C-*myb* (Ratajczak). In most of these trials, PS-oligonucleotides have been administered intravenously. However, in the cases of HPV and HCMV, PS-oligonucleotides have been administered intradermally and intravitreally, respectively.

For the treatment of patients with concurrent HIV-1 and HCMV infection, doses of 75 to 300 μg of 21-mer PS-oligonucleotides were administered each week for four weeks, and then every other week. Intravitreal injections once a week for 2–3 weeks produced the best results in terms of safety, tolerance, and control of replication of HCMV.

For the treatment of HIV-1, PS-oligonucleotide was administered to a range of patients by two-hour long intravenous infusions at doses ranging between 0.1 and 2 mg kg^{-1} body weight. In one study, ^{35}S-labeled PS-oligonucleotide was administered to six individuals by two-hour long infusion at a dose of 0.1 mg kg^{-1} body weight. Plasma half-lives were 0.18 ($t_{\frac{1}{2}}\alpha$ – rapid elimination) and 26.7 ($t_{\frac{1}{2}}\beta$ – slower elimination) hours. Most of the PS-oligonucleotide was excreted in the urine, with 49% of the administered dose excreted by 24 hours, and 70% by 96 hours.

In another clinical trial, PS-oligonucleotide against *p53* was administered to patients with acute myelogenous leukemia (AML) that is associated with myelodysplastic syndrome (MDS). The PS-oligonucleotide was administered for ten days by continuous intravenous infusion, at doses of between 0.05 and 0.75 mg kg^{-1} hr^{-1}. The peak plasma concentration and the area under the plasma concentration curve were linearly proportional to the dose. The elimination rate increased as a function of dose from 24.4% at 0.05 mg kg^{-1} hr^{-1} to 62.5% at 0.25 mg kg^{-1} hr^{-1}.

Modification of oligonucleotides

It has been established by studies carried out to date that the pharmacokinetic and pharmacodynamic properties of PS-oligonucleotides are largely independent of sequence. However, these properties are dependent on the nature of the backbone of the oligonucleotide. In an attempt to modulate these properties, chemists are designing various oligonucleotides by modifying the backbone, either by combining two different backbones in one oligonucleotide sequence, forming chimeric oligonucleotides (Agrawal), or by combining segments of PS-oligonucleotide and modified RNA (Agrawal and Ecker). These modified oligonucleotides have shown improved antisense activity against Ha-*ras* (Ecker), HIV-1 and influenza virus (Agrawal). In pharmacokinetic studies, PS-oligonucleotides containing segments of either modified DNA or RNA were located in different tissues, and had significantly improved *in vivo* stability over unmodified oligonucleotides. An increase in *in vivo* stability may provide longer duration of action, leading to a decrease in the frequency of administration. Preliminary studies suggest that intact modified oligonucleotides are bioavailable after oral administration, probably because they are stable at low pH, and are resistant to nucleases (Agrawal).

Ribozyme therapy

Ribozymes are RNA sequences (antisense oligonucleotides) that can catalyze biochemical reactions, inactivating the target sequence and releasing the oligonucleotide to move to another target molecule (John Rossi, Loma Linda University School of Medicine, Loma Linda, CA, USA). The two most widely studied ribozyme motifs are the 'hammerhead' and the 'hairpin', which cleave the target at a GUC recognition site.

Rossi's laboratory has demonstrated in cell culture the anti-HIV

activity of a hammerhead ribozyme targeted to conserved sites in the *tat*, and a shared *tat–rev*, exon. These ribozymes were also effective at preventing HIV infection of stem cells challenged with HIV-1.

As is the case for antisense oligonucleotides, ribozymes must reach the appropriate intracellular sites of action in order to achieve optimal biological activity. Rossi has demonstrated a 4–12-fold improvement in anti-HIV efficacy using an intracellular targeting strategy that involves attaching a tRNA Lys3 primer site for reverse transcriptase to the 5′-end of the hammerhead ribozyme. This acts as a vehicle for carrying the ribozyme close to the HIV genomic RNA, and possibly into the virion itself. With improved intracellular targeting and catalytic activity, ribozymes may prove to be a powerful tool for modulating gene expression.

In summary, first generation antisense oligonucleotides have reached the human clinical trial stage. Using accumulated experience, various groups are trying to improve the pharmaceutical properties of oligonucleotides.

The antisense oligonucleotide approach provides significant advantages over other rational drug design approaches, because it provides the flexibility of incorporating various chemical modifications, to modulate various pharmaceutical properties, without affecting the interaction with the disease-associated gene.

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